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Extracellular Metabolism of Sucrose in a Submerged Culture of *Claviceps purpurea*: Formation of Monosaccharides and Clavine Alkaloids

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Transformation of extracellular sucrose during cultivation of *Claviceps purpurea* led to the formation of mono- and oligosaccharides. Maltose was a suitable substrate for submerged fermentation of alkaloids. Fermentation in a medium with maltose was characterized by an insignificant formation of glucans, intensive sporulation, suspension growth of mycelium, and a higher formation of elymoclavine. Glucose alone yielded low levels of total alkaloids and high glucan formation; on the other hand, glucose promoted the formation of elymoclavine.

Sucrose is a dominant component of the phloem sap of plants infected by the fungus *Claviceps purpurea* (3). It is also a usual component of media for the cultivation of saprophytic strains of *C. purpurea* and media for fermentation of ergot alkaloids. During the cultivation of the fungus, extracellular sucrose undergoes changes due mostly to exocellular β-D-fructofuranosidase (6, 12). The transfructosylase activity of this enzyme plays a prominent role in the formation of oligosaccharides (13), which are further transformed by transfructosylation (6). β-D-Fructofuranosidase is also capable of transferring fructosyl residues from sucrose to methanol, ethanol, and elymoclavine (generally to primary alcoholic groups [10]) with the formation of methyl-, ethyl- (12), or elymoclavine-*O*-β-D-fructofuranoside (9).

Transformation of sucrose yields mostly glucose, whereas most fructose is incorporated into oligosaccharides. Glucose is utilized by the organism immediately, whereas fructose is utilized only after glucose depletion. Free fructose inhibits growth, and in a 5% concentration it totally inhibits sporulation (6).

Oligosaccharides containing 70 to 80% fructose remain in the medium and are metabolized at later stages of fermentation. Oligosaccharides can be considered reserve substance (1).

Extracellular β-D-glucan is another reserve nutrient. This polysaccharide consists of a β-D-(1→3) backbone with 25% of β-D-(1→6) branch points (5, 14). Glucans are mostly synthetized at the beginning of cultivation and are degraded at later stages (7). The cell wall of *Claviceps paspali* also contains glucans with β-D-(1→4) and β-D-(1→6) bonds. Deposition of polysaccharides into the cell walls is proportional to the intensity of formation of ergot alkaloids (20).

Extracellular metabolism of sucrose has so far been studied mainly with regard to the formation and structure of poly- and oligosaccharides. We focused our attention on the occurrence of mono- and disaccharides, which function as direct substrates and effectors of culture growth and in some cases as physiological indicators.

MATERIALS AND METHODS

Strain and media. *C. purpurea* 129/35 producing clavine alkaloids was obtained by UV light mutagenesis and is deposited in the collection of the Institute of Microbiology, Prague, Czechoslovakia.

The inoculum for submerged culture was prepared in medium T1 as described earlier (11). Submerged fermentation was carried out in 300-ml Erlenmeyer flasks with 60 ml of CS2 medium (19). CMG medium was a modification of CS2 medium and contained the following ingredients (in grams per liter): maltose, 95; glucose, 5; citric acid, 16.8; $CaCl_2$, 1.2; $(NH_4)_2SO_4$, 0.25; $MgSO_4 \cdot 7H_2O$, 0.25; KCl, 0.12; $FeSO_4 \cdot 7H_2O$, 0.02; $ZnSO_4 \cdot 7H_2O$, 0.015; and distilled water, pH 5.5 (NaOH).

Analytical methods. The dry weight of mycelia was determined gravimetrically (11). Total clavine alkaloids were determined with the van Urk reagent (17). The spectrum and qualitative proportion of alkaloids were determined by high-pressure liquid chromatography (11). Total saccharides in the fermentation medium were determined by the anthrone method (4). Extracellular glucans were determined gravimetrically after precipitation with a fourfold volume of acetone and drying (4 h at 70°C). Each culture sample was observed in a light microscope. The level of glucose was determined by gas-liquid chromatography and verified by the glucose oxidase method, using the Bio-La-Test set (Lachema, Brno, Czechoslovakia). The analysis of mono- and disaccharides was carried out by gas chromatography. Samples of the medium were dried by lyophilization at −20°C and were further dried in vacuo above P_2O_5. The medium dry weight was determined at the same time. Powdered samples were derivatized and analyzed by a modification (21) of the method of Sweeley et al. (18). Silylated mixture was analyzed on a Perkin-Elmer Sigma 3B gas chromatograph with a glass column (2,000 by 4 mm) filled with 2.5% SE-52 (General Electric Co., Schenectady, N.Y.) on Chromosorb G-AW-DMCS (particle size, 0.15 to 0.17 mm; Johns Manville, Denver, Colo.). The temperature program of the column was 160 to 300°C with a linear gradient 3°C/min and a subsequent isothermic regime at 300°C. The temperature of the flame ionization detector was 320°C, and the injector temperature was 230°C. The carrier gas was N_2 (25 ml/min), and the sample volume was 1 to 3 μl. The internal standard in quantitative determinations of saccharides was ribose (Fluka, Buchs, Switzerland). Standard solutions of trimethylsilyl derivatives of saccharides in

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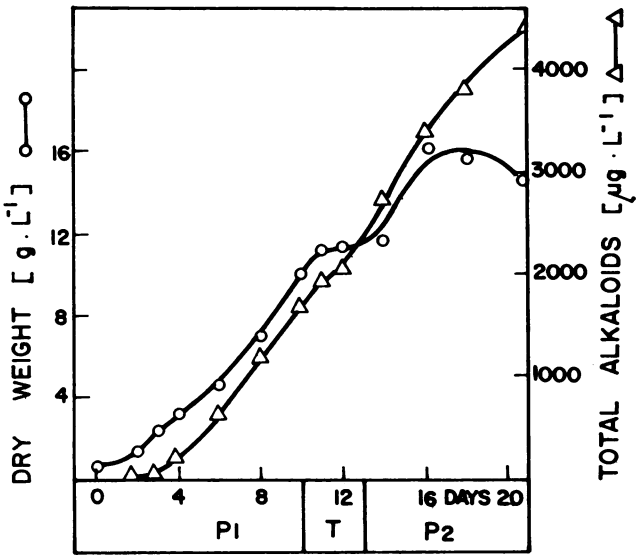

FIG. 1. Production and growth phases (P1, T, and P2) of a submerged culture of *C. purpurea* 129/35.

hexane were from Pierce Eurochemie, Rotterdam, The Netherlands; other chemicals were from Lachema.

RESULTS

Submerged culture of *C. purpurea* 129/35 produced agroclavine (70 to 80%), elymoclavine (15 to 30%), chanoclavine-I as a minor product, and other clavine alkaloids in traces. The formation of alkaloids had two production phases (P1 and P2) and a transition phase (T) (Fig. 1). Phase T exhibited an obvious lag in the culture growth with a subsequent lowering of the intensity of alkaloid production.

Formation of glucans occurred in phase P1. In phase T, this formation ceased, and glucans were intensively degraded.

Total saccharides were drawn from the medium at a high rate at the beginning of fermentation (Fig. 2). In phase T, their utilization slowed down and almost ceased, whereas in phase P2 a part of the carbohydrates was used for secondary growth. The initial fast utilization of saccharides, which leaves a 20 to 30% nonmetabolized residue, is a typical phenomenon in fermentation of ergot alkaloids (7, 14).

The sharp drop of sucrose content in the medium at the beginning of fermentation was largely due to β-D-fructofuranosidase. The decrease in sucrose level correlates with the liberation of its components, glucose and fructose (Fig. 3). For the most part, fructose is directly incorporated into oligosaccharides. These processes, and especially the subsequent secondary transformations of oligosaccharides, are accompanied by a slight release of fructose into the medium (6).

In contrast to fructose, glucose appeared in the medium at the beginning of fermentation in a substantially higher concentration. It was rapidly utilized by the culture and thus probably participated in the development of invertase activity (12). Beginning with day 12, the concentration of glucose was 0.07 g/liter on the average.

Besides glucose and fructose, other monosaccharides were also found in the medium. Of the pentoses, xylose (0.15 to 0.45 g/liter) was found in phases P1 and T in parallel with traces of galactose and mannitol (0.1 to 0.4 g/liter each). Mannose appeared in large amounts (Fig. 3); its level during cultivation exhibited two maxima, the first in phase P1 when spore germination took place along with extensive proliferation and differentiation of cells, and the second in phase P2. In phase T, when the culture growth almost stopped, the concentration of mannose in the medium decreased. Towards the end of cultivation (day 21), mannose was released into the medium from autolyzing mycelia.

Disaccharides found in the fermentation medium included α and β-maltose (0.3 to 0.55 g/liter), and their levels were highest (1.3 g/liter) in phase T, i.e., on day 11. Smaller amounts of trehalose (0.05 to 0.2 g/liter), a characteristic saccharide in the sclerotia of the parasitic form of *C. purpurea* (13), were also found in the medium. Unidentified peaks D I and D II with retention times shorter than that of sucrose (Fig. 4) were attributed to difructans arising in secondary transformation reactions from oligosaccharides. Both saccharides occurred in the medium during fermentation in substantial amounts, the highest concentrations being found at 48 h of fermentation (D I, 3.7 g/liter; D II, 8.0 g/liter).

In fermentation medium with 10% sucrose, the strain under study produced 2,000 to 4,000 μg of total alkaloids per ml, predominantly agroclavine (85%). On the other hand, in medium with 10% glucose, the production of the alkaloids was much lower (300 μg/ml), but the dominant component of the alkaloids was elymoclavine (85%).

Another saccharide tested as a component of the fermentation medium was maltose. With regard to the intensity of culture growth and production of total alkaloids, media with maltose (CMG) and sucrose (CS2) were nearly equal. In other respects, however, CMG medium was more suitable than CS2 medium. On CMG medium, the yield of the desirable elymoclavine (20) was higher (907 μg/ml) than on CS2 medium (338 μg/ml). Culture in CMG medium formed only traces of extracellular glucans, unlike culture in CS2 medium (21 g of glucan per liter in 20-day-old culture). After 5 to 6 days, the CMG culture split into arthrospores and assumed suspension growth. This pattern contributes substantially to improved bioengineering parameters of fermentation of clavine alkaloids.

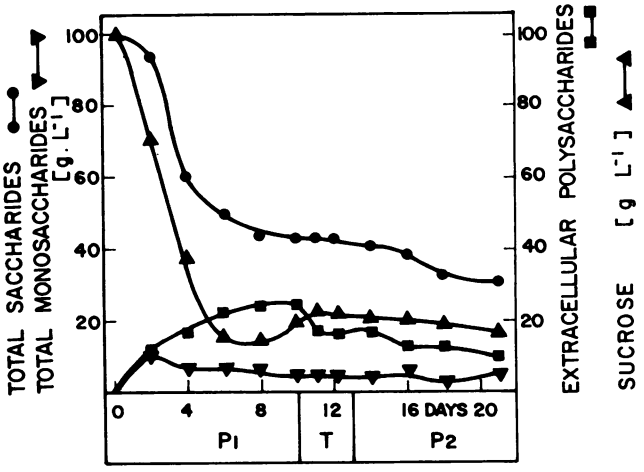

FIG. 2. Sucrose metabolism in a submerged culture of *C. purpurea* 129/35.

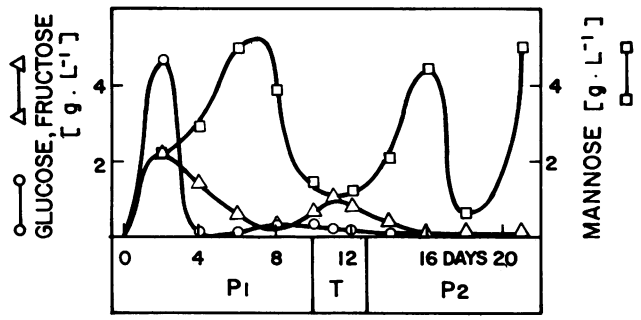

FIG. 3. Content of glucose, fructose, and mannose in the culture medium during submerged cultivation of *C. purpurea* 129/35.

DISCUSSION

Production of clavine alkaloids is always associated with an intensive assimilation of sucrose (12). Extracellular sucrose undergoes complex transformations before its ultimate utilization by cells. These transformations are species specific. For instance, *C. purpurea* splits sucrose with β-D-fructofuranosidase, and *C. paspali* splits it with endosucrase (19). Transformations of sucrose and their product affect the growth and development of the culture, in some cases simply because of osmolarity changes (15). They also intervene significantly in the course of fermentation, e.g., via changes in rheological properties of the medium (glucans) and subsequently also via changes in other bioengineering parameters, especially oxygen transfer.

The enzyme complex of *C. purpurea* degrades sucrose and is also probably capable of resynthesizing it in some phases of fermentation (Fig. 2). The hypothesis of sucrose resynthesis is supported by the saccharide balance and the increase in sucrose level in phase T. In this phase, the level of glucans decreased, that of free fructose transiently rose (Fig. 3), and the level of total monosaccharides remained relatively constant. Glucose, liberated by the splitting of extracellular glucans, affects the invertase activity in the direction of sucrose resynthesis (8).

The difference in the resynthesis of sucrose in molds and yeasts indicates a difference between the invertases of these two types of organisms. With glucose as an acceptor, mold invertase produces sucrose, whereas yeast invertase gives rise only to 6-fructofuranosylglucose (10).

The activity of exocellular β-glucosidases in the transition phase T increases (S. Pažoutová, Ph.D. thesis, Institute of Microbiology, Prague, Czechoslovakia, 1980). The splitting of glucans is ascribed to constitutive β-D-1,3-glucanases and β-glucosidases. Constitutive glucanases are commonly found in almost all fungi (16). In *C. purpurea*, glucanases and glucosidases appear at the moment of differentiation of mycelium to sclerotial cells (7). According to Dickerson et al. (7), the synthesis of glucans is associated with sphacelial cells, and glucan splitting is associated with sclerotial cells. The extensive rearrangment of poly- and oligosaccharides in phase T is probably connected with the occurrence of a higher amount of maltose in this period.

Except during the initial phases of cultivation, the concentration of glucose during the fermentation was low, this implies that the glucose liberated during sucrose transfructosylation is intensively utilized by the organism or incorporated into glucans.

A remarkable phenomenon was the occurrence of a substantial amount of extracellular mannose in the medium during alkaloid fermentation (Fig. 3). Mannose is found in the form of mannoproteins in the cell walls of yeasts and fungi (2) and is liberated into the medium during growth and remodeling of the cell wall. These facts, as well as the results obtained with the submerged culture of *C. purpurea*, lend support to the two-phase production scheme with a second-

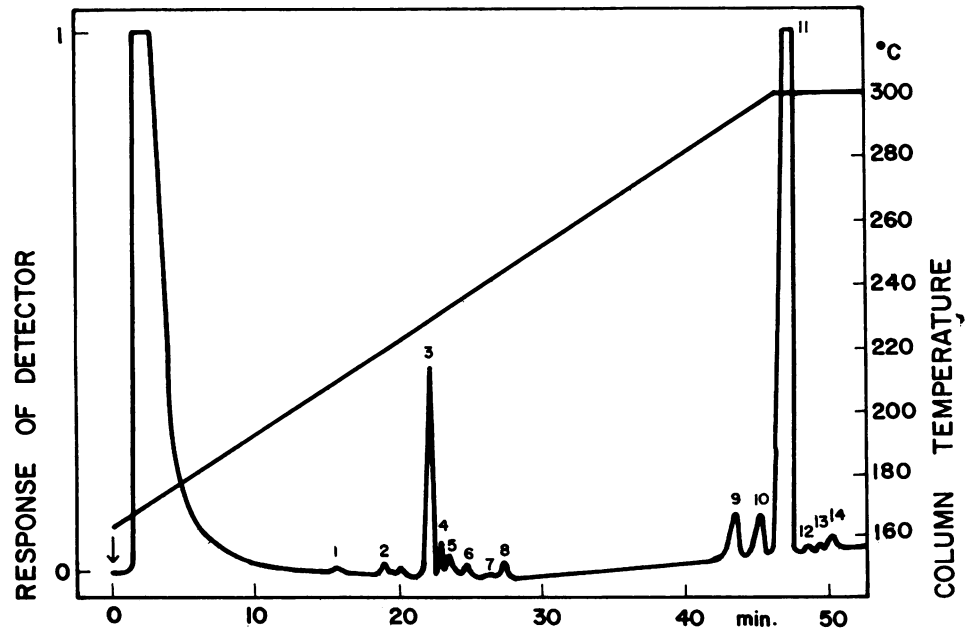

FIG. 4. Saccharides from the fermentation medium on day 11 of cultivation. Peaks are numbered as follows: 1, rhamnose; 2, xylose; 3, mannose; 4, fructose; 5, galactose; 6, α-glucose; 7, mannitol; 8, β-glucose; 9, D I; 10, D II; 11, sucrose; 12, α-maltose; 13, trehalose; 14, β-maltose.

ary growth and germination of spores in phase P2. Mannose can also be classified as a suitable physiological indicator of growth intensity of *C. purpurea* cultures and of intensity of alkaloid production.

Our results point out that the view that glucose is an unsuitable substrate for high-production fermentation should be revised. Glucose is the most readily utilizable substrate, and in a sucrose medium the cells grow essentially on glucose. The inhibition of alkaloid formation takes place only at high glucose concentrations. Glucose, or some of its related metabolites, act also as a feed-forward regulator of phosphofructokinase (V. Křen and Z. Řeháček, Speculations Sci. Technol., in press). At the same time, a high content of glucose stimulates the production of undesirable glucans. The composition of cultivation medium can be optimized by using the following mechanism of sucrose transformations: glucose is released into the medium gradually and is immediately metabolized so that the repressive concentration is never exceeded (Fig. 3). A surplus of glucose is obviously incorporated into inert glucans. Fructose, which is substantially less suitable as a growth substrate, is transiently put out of the way by incorporation into oligosaccharides. A more suitable substrate would be an easily available disaccharide composed solely of glucose units and readily split by *C. purpurea* glucosidases. These demands are met by maltose in view of the proved maltase activity of *C. purpurea* (13). CMG medium made it possible to maintain high yields of total alkaloids and at the same time to increase the production of elymoclavine and improve the rheological properties of the culture.

From other preliminary experiments with glucose and sucrose, it can be assumed that constant glucose level lower than that which reduces dramatically the total amount of alkaloids would support production of elymoclavine in a significant amount, i.e., both a high production of total alkaloids and a high percentage of elymoclavine. The effect of maltose could then be ascribed to the slow liberation of glucose maintaining its constant level during the whole fermentation. Better results might be obtained by controlled feeding of glucose into the fermentation tank. The problem is still under study.

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LITERATURE CITED

1. **Arcamone, F., G. Cassinelli, G. Ferni, S. Penco, P. Penella, and C. Pol.** 1970. Ergotamine production and metabolism of *Claviceps purpurea* strain 275 FI in stirred fermenters. Can. J. Microbiol. **16**:923–931.
2. **Ballou, C. E.** 1982. Yeast cell wall surface, p. 335–360. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Basset, R. A., E. B. Chain, A. G. Dickerson, and P. G. Mantle.** 1972. Comparative metabolism of *Claviceps purpurea* in vivo and in vitro. Biochem. J. **127**:3P–4P.
4. **Beink, R. H., Jr., P. Duback, and D. L. Lynch.** 1960. Measurements of carbohydrate in soil hydrolysates with anthrone. Soil Sci. **89**:157–166.
5. **Buck, K. W., A. W. Chen, A. G. Dickerson, and E. B. Chain.** 1968. Formation and structure of extracellular glucans produced by *Claviceps* species. J. Gen. Microbiol. **51**:337–352.
6. **Dickerson, A. G.** 1972. A β-D-fructofuranosidase from *Claviceps purpurea*. Biochem. J. **129**:263–272.
7. **Dickerson, A. G., P. G. Mantle, and C. A. Szczyrbak.** 1970. Autolysis of extracellular glucans produced in vitro by a strain of *Claviceps fusiformis*. J. Gen. Microbiol. **60**:403–415.
8. **Edelman, J.** 1953. Transfer reactions catalysed by some sucrase preparations. Biochem. J. **57**:22–33.
9. **Floss, H. G.** 1976. Biosynthesis of ergot alkaloids and related compounds. Tetrahedron **32**:873–912.
10. **Myrbäck, K.** 1960. Invertases, p. 379–393. *In* P. D. Boyer, H. Lardy, and K. Myrbäck (ed.), The enzymes, vol. 4. Academic Press Inc., New York.
11. **Pažoutová, S., M. Flieger, P. Sajdl, Z. Řeháček, J. Taisinger, and A. Bass.** 1981. The relationship between intensity of oxidative metabolism and predominance of agroclavine or elymoclavine in submerged *Claviceps purpurea* cultures. J. Nat. Prod. (Lloydia) **44**:225–235.
12. **Perényi, T., E. Udvardy-Nagy, and E. K. Novák.** 1968. Studies on sucrose breakdown by invertase-producing *Claviceps* strains. Acta Microbiol. Acad. Sci. Hung. **15**:17–28.
13. **Perényi, T., E. Udvardy-Nagy, G. Wack, and E. K. Novák.** 1966. Oligosaccharide utilization by saprophytic *Claviceps* strains. Acta Microbiol. Acad. Sci. Hung. **13**:205–214.
14. **Perlin, A. S., and W. A. Taber.** 1963. A glucan produced by *Claviceps purpurea*. Can. J. Chem. **41**:2278–2282.
15. **Puc, A., and H. Sočič.** 1977. Carbohydrate nutrition of *Claviceps purpurea* for alkaloid production related to the osmolarity of media. Eur. J. Appl. Microbiol. **4**:283–287.
16. **Reese, E. T., and M. Mandels.** 1959. β-D-1,3-Glucanases in fungi. Can. J. Microbiól. **5**:173–185.
17. **Robbers, J. E., L. W. Robertson, K. M. Hornemann, A. Jindra, and H. G. Floss.** 1972. Physiological studies on ergot: further studies on the induction of alkaloid synthesis by tryptophan and its inhibition by phosphate. J. Bacteriol. **112**:791–796.
18. **Sweeley, C. C., R. Bentley, M. Makita, and W. W. Wells.** 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Am. Chem. Soc. **85**:2497–2507.
19. **Tonolo, A., and E. Udvardy-Nagy.** 1968. Production of clavine-alkaloids by *Claviceps fusiformis* (Loveless) in submerged culture. Acta Microbiol. Acad. Sci. Hung. **15**:29–40.
20. **Vořišek, J., J. Ludvík, and Z. Řeháček.** 1974. Cytochemical localization of polysaccharides in *Claviceps paspali*. Ultrastructure during submerged fermentation of alkaloids. J. Bacteriol. **118**:285–294.
21. **Wurst, M., K. Sigler, and A. Knotková.** 1980. Gas chromatographic determination of extracellular metabolites produced by baker's yeast during glucose-induced acidification. Folia Microbiol. **25**:306–310.